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Identification and pathogenicity of fungal species associated with leaf spot of mango trees in the south of Iran

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Abstract

During the survey of mango (*Mangifera indica*, *Anacardiaceae*) trees in Sistan and Baluchestan, Hormozgan, and Kerman Provinces, the occurrence of leaf spot diseases was observed. Symptomatic samples were collected and subsequently transferred to the laboratory for the purpose of isolating the causal agents. After surface sterilization of selected leaf tissues, small leaf pieces were transferred to potato dextrose agar (PDA). In order to achieve purification, single spore or hyphal tip methods were used. Based on morphological criteria and analyzing data from the ITS-rDNA and glyceraldehyde 3-phosphate dehydrogenase (*gpd*) gene regions, 118 isolates belonging to *Alternaria alternata*, *Curvularia hawaiiensis*, and *Exserohilum rostratum* species were identified. Pathogenicity test was carried out by using detached leaf assay on leaf pieces of mango. After seven days, necrotic lesions were observed on inoculated leaves and fungal isolates were re-isolated and identified to confirm Koch's postulates. To the best of our knowledge, This study is the first report of *C. hawaiiensis* and *E. rostratum* as the causal agents of leaf spot on mango trees in Iran.

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Introduction

The popularity of mango as a tropical fruit has grown significantly, leading to its cultivation in both traditional and non-traditional production regions (Berardini et al., 2005; Nelson, 2008; Saeed et al., 2017). The southern regions of Iran, encompassing substantial portions of Hormozgan and Sistan and Baluchestan Provinces, are widely regarded as the most optimal areas within the nation for the cultivation of tropical commodities (Saboki et al., 2012, 2014).

Mango trees encounter a diverse range of pathogens that impact every component of the tree, consequently diminishing the global fruit yield and quality (Prakash, 2003). The mango fruit is highly beneficial due to its abundance of essential nutrients, namely vitamins A, B, C, and K. Additionally, its delectable pulp and captivating aroma further contribute to its appeal (Shih-Lun et al., 2020). Although the mango fruit is susceptible to a considerably broad range of diseases, some diseases possess significant economic relevance and are accountable for substantial losses in mango cultivation in some countries (Misra, 2011). Leaf spot diseases are significant foliar diseases found in tropical fruit trees, leading to economic loss in these host plants (Okigbo & Osuinde, 2003).

In Iran, *Alternaria alternata*, *A. destruens*, *Aspergillus niger*, *Fusarium semitectum*, *Gibberella intricans* (Karampour & Ershad, 2008), *Botryodiplodia mangiferae* (Ershad, 2009), *Botryosphaeria rhodina* (Mirzaee et al., 2002), *Dothiorella ladharensis* (Petrak, 1956), *Glomerella cingulate* (Zebarjad & Ershad 2008), *Oidium mangiferae* (Zakii et al., 1993), *Bartalinia pini* and *Beltrania rhombica* (Dehghani et al., 2022) have been reported as the causal agents of leaf spot disease on mango. Here, we focused on leaf spot diseases of mango trees in the south of Iran. We isolated 118 fungal isolates of *Alternaria*, *Curvularia*, and *Exserohilum* genera, and a pathogenicity test was conducted on mango leaves.

Material and Methods

Sampling and fungal isolation

Mango leaves exhibiting leaf spot symptoms were collected from various regions of Sistan and Baluchestan, Hormozgan, and the southern region of Kerman Provinces. The initial isolation process involved the cutting of the infected leaves into pieces measuring 7-8 mm. Following this, surface disinfection was carried out employing a 1% solution of sodium hypochlorite for three minutes. Subsequently, the leaf segments were rinsed three times with sterile distilled water (SDW) and plated on 2% water-agar (2% WA). Plates were incubated at 25°C in the dark. Purified isolates were acquired through the transfer of the produced conidia on 2% water-agar (2% WA) and moist filter paper at a temperature of 25°C to Potato Dextrose Agar (PDA) medium using both single spore and hyphal tip methods (Pordel et al., 2015).

Identification of the fungal isolates

Morphological criteria

The pure isolates of *Curvularia* and *Exserohilum* were plated on PDA, tap water agar amended with autoclaved wheat straw (TWA–wheat straw) medium and incubated at 25°C. Subsequently, All plates were incubated at 25 ± 2°C under near UV light (NUV) with a 12 h photoperiod, over the course of two weeks (Sivanesan, 1987, Hernández-Restrepo et al., 2018). *Alternaria* isolates were identified based on their morphological characteristics. Purified cultures were transferred to potato carrot agar (PCA) and incubated at 23-25°C under an 8/16-hour light/dark cycle photoperiod for five to seven days (Ellis, 1971; Simmons, 2007). Subsequently, the colony morphology and microscopic features were scrutinized, measured, and documented. The colony color was determined according to the color charts developed by Rayner (Rayner, 1970). The pure cultures were deposited in the Herbarium of the Mycology Laboratory at the University of Jiroft, Kerman (UJFCC).

Molecular identification

Nine isolates (three isolates for each species) were selected for molecular studies. Total genomic DNA of the isolates was extracted according to Cenis (Cenis, 1972). The PCR program was employed for amplifying the internal transcribed spacer (ITS-rDNA) with ITS1 and ITS4 primers (White et al., 1990) and glyceraldehyde 3-phosphate dehydrogenase (*gpd*) with gpd1 and gpd2 primers (Berbee et al., 1999) regions consisted of an initial denaturation at 94°C for three minutes, followed by 35 cycles of 94°C for 30 seconds, 55°C for 50 seconds, 72°C for one minute, and a final extension at 72°C for 10 minutes (Lin et al., 2017). Polymerase chain reaction (PCR) products were visualized on a 1.5% agarose gel to validate the presence and size of amplicons. The amplified products underwent sequencing by Microsynth Inc. (Switzerland). The quality of the sequences was assessed by checking the chromatograms using BioEdit version 7.2 (Hall, 1999). Subsequently, the sequences derived from the amplification of a portion of the genomic region were compared with those in the GenBank using the BLASTn search tool, and the modified sequences were submitted to NCBI GenBank.

Pathogenicity tests

After accurate species identification, three isolates belonging to the species *Alternaria alternata* UJFCC1992, *Curvularia hawaiiensis* UJFCC2068, and *Exserohilum rostratum* UJFCC2074 were selected as representatives for pathogenicity tests on detached leaves of mango. Isolates were cultivated on tap water agar containing autoclaved wheat straw (TWA-wheat straw) and PCA medium and incubated at 25°C for two weeks according to the aforementioned conditions. The conidia were harvested and subsequently rinsed with 3–5 ml of sterile distilled water. The concentration of conidia was determined using a hemocytometer and adjusted to 2×10^5 spores/ml-1 for the purpose of inoculation. Following that, the mango leaves were subjected to a 10 ml conidia suspension spray, and the control leaves were treated with a 10 ml spray of sterile distilled water. Infected leaves were wet in the substrate for seven days (Shi et al., 2012). The pathogenicity test was repeated three times.

Results

In this study, 118 fungal isolates belonging to *Alternaria alternata* (72 isolates; 32 isolates from Sistan and Baluchestan, 26 isolates from Hormozgan and 14 isolates from Kerman), *Curvularia hawaiiensis* (35 isolates; 22 isolates from Sistan and Baluchestan, nine isolates from Hormozgan and four isolates from Kerman), and *Exserohilum rostratum* (11 isolates; five isolates from Sistan and Baluchestan and six isolates from Hormozgan) were isolated from mango trees showing leaf spot in the south and southeast of Iran.

Morphological and molecular criteria

***Alternaria alternata* (Fr.) Keissl., Beih. bot. Zbl., Abt. 2 29: 434 (1912)**

The colony on PCA exhibited an initial whitish coloration with the presence of aerial mycelium, subsequently transitioning from an olivaceous green hue to a darker green. Conidiophores branched, smooth, straight, golden brown, up to 10-80 µm long and 3–4 µm thick. Conidia were produced in long branched chains, golden brown to dark brown, ellipsoidal or obpyriform, 7–38 × 7–12 µm, with 2–7 transverse septa and 1–3 longitudinal septa. These characteristics matched well with the description of *Alternaria alternata* (Ellis, 1971; Simmons, 2007) (Fig. 1). The comparison of the *gpd* sequences revealed a 98% similarity to the type strain of *A. alternata* (strain SF-005; GenBank accession no. ON055384). Therefore, based on morphological, cultural, and molecular data, the isolates were identified as *A. alternata*.

Alternaria alternata is an opportunistic fungal pathogen that has been recorded on more than 100 host species and causes several diseases such as; leaf spots, rots, and blights on many plant parts (Agrios, 2005). Morphologically, *A. alternata* is distinguishable from other *Alternaria* species by its conidia branched chains (Simmons, 2007; Woudenberg et al., 2013). It has been reported on mango in Hormozgan Province (Ershad, 2009), and this is the first report of leaf spot caused by this species on mango in south of Kerman and Sistan and Baluchestan Province.

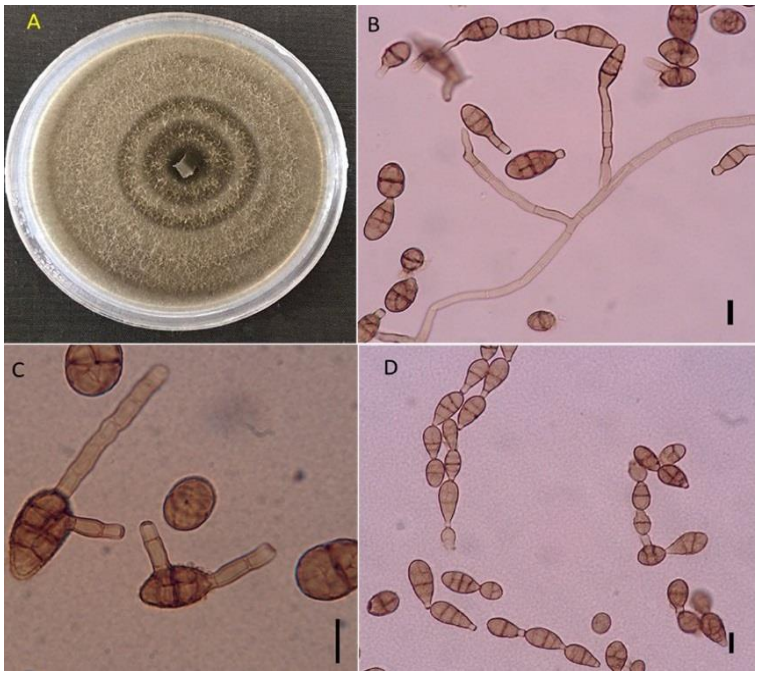

Fig. 1. *Alternaria alternata* (A) A seven-day-old colony on PDA, (B) Conidiophores, (C-D) Conidia. Scale bar=10 µm.

***Curvularia hawaiiensis* (Bugnic. ex M.B. Ellis) Manamgoda, L. Cai & K.D. Hyde, Fungal Diversity 56: 141**

The colony displayed a hue of olivaceous green on its surface and a dark green hue on the underside when cultivated on PDA. Conidiophores single, flexuous, geniculate, pale brown to brown, 20–255 × 2–4 µm. Conidiogenous nodes were dark brown and verruculose. Conidia ellipsoid, oblong or cylindrical, rounded at ends, pale to mid brown, 13–34 × 5–10 µm, 4–5-distoseptate, often germinating by a germ tube from one end (Fig. 2). Morphology of the specimen examined agrees with the description provided by Ellis (1971). The ITS sequence (GenBank accession no. MN053866) comparison showed 99% identity to *C. hawaiiensis* type strain (BRIP 11987, GenBank accession no. KJ415547). According to morphological and molecular data, our isolates were identified as *C. hawaiiensis*.

Regarding the recent reclassification of *Bipolaris* by Mananmgoda et al. (2014), this species was transferred from *Bipolaris* to *Curvularia*. It is morphologically very close to *Bipolaris homomorphus* but is distinctly characterized by its cylindrical conidia. Ghasemi-Sardareh & Mohammadi (2020) previously reported *C. hawaiiensis* from neem (*Azadirachta indica*) trees in Iran, and this is the first report of pathogenicity of *C. hawaiiensis* on mango trees in Iran.

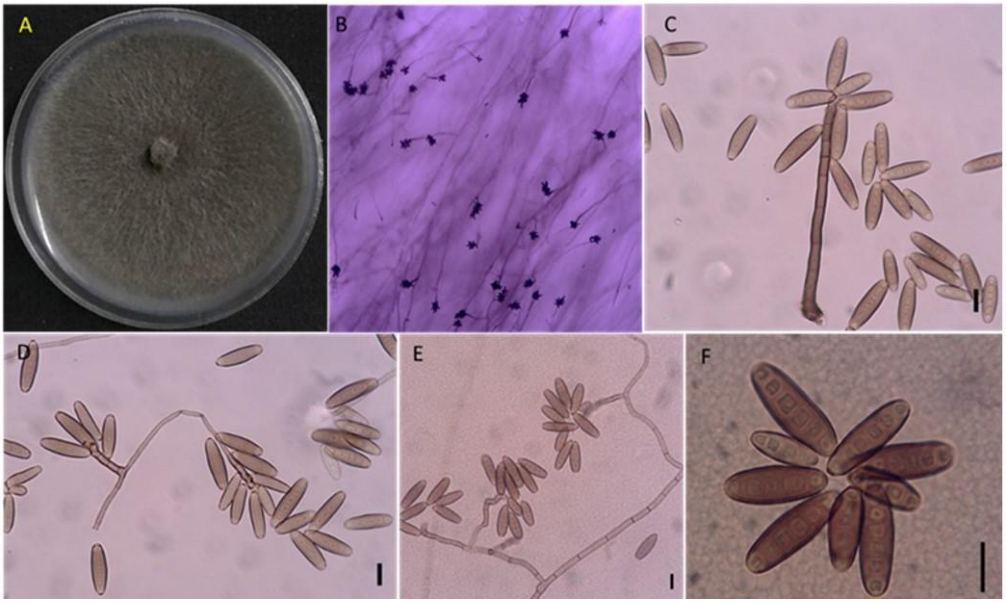

Fig. 2. *Curvularia hawaiiensis* (A) A seven-day-old colony on PDA, (B) Habit of conidiophores, (C-E) Conidiophores (F) Conidia. Scale bar=10 µm.

***Exserohilum rostratum* (Drechsler) K.J. Leonard & Suggs, Mycologia 66: 290 (1974)**

The colony was olivaceous brown on PDA. Conidiophores single, cylindrical, geniculate, dark brown, up to 100 µm long. Conidia straight to slightly curved, ellipsoidal to narrowly obclavate or rostrate, brown, 3–16 distoseptate, basal septum darker and thicker than other septa, 14–34×8–11 µm, with a distinctly protruding basal hilum. Germination of conidia is monopolar and bipolar (Fig. 3). The specimens examined in this study had the same characteristics of *E. rostratum* as described by Sivanesan (1987) and Hernandez-Restrepo et al. (2018). Based on morphological characteristics, the isolates were identified as *E. rostratum*.

As mentioned by Sivanesan (1987), *E. rostratum* is a commonly encountered opportunistic fungus, previously reported on some plants in Iran (Ershad, 2009), but to our current knowledge, this is the first report of *E. rostratum* as the causal agent of leaf spot disease on *M. indica* in Iran.

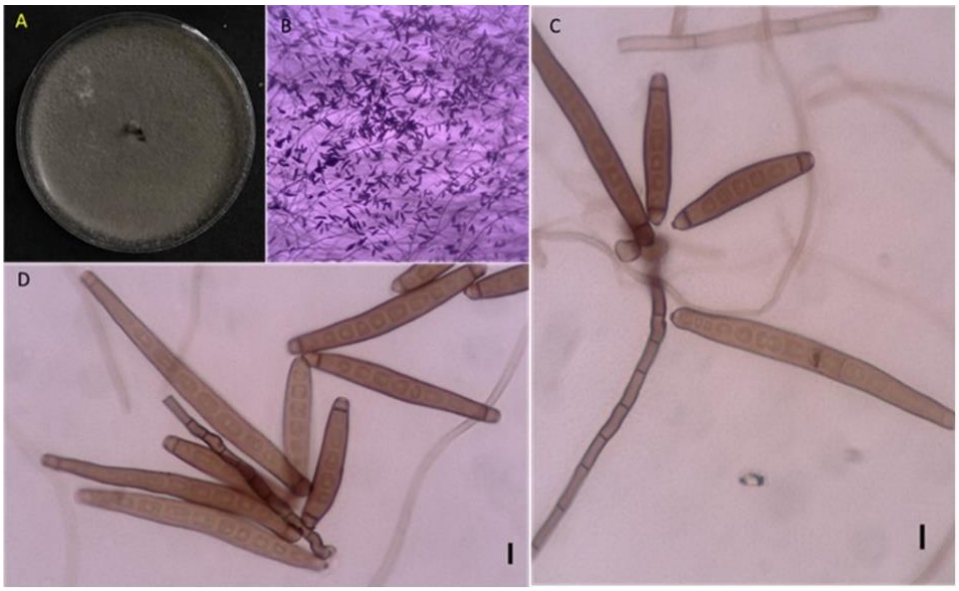

Fig. 3. *Exserohilum rostratum* (A) A seven-day-old colony on PDA, (B) Habit of conidiophores, (C-D) Conidia and Conidiophores. Scale bar=10 µm.

Pathogenicity test

The symptoms of the disease were reproduced on the leaf fragments of mango seven days after inoculation by *Alternaria alternata*, *Curvularia hawaiiensis*, and *Exserohilum rostratum*. The appearance of symptoms occurred within a span of five days following inoculation, characterized by red-dot symptoms initially and progressing to necrotic symptoms after seven days (Fig. 4; A-C). Fungal isolations were conducted from the necrotic tissues, and subsequent re-isolation of *A. alternata*, *C. hawaiiensis*, and *E. rostratum* isolates verified their identical nature to those employed in the pathogenicity assay. No fungi were re-isolated from the control treatments and symptoms did not manifest in the control.

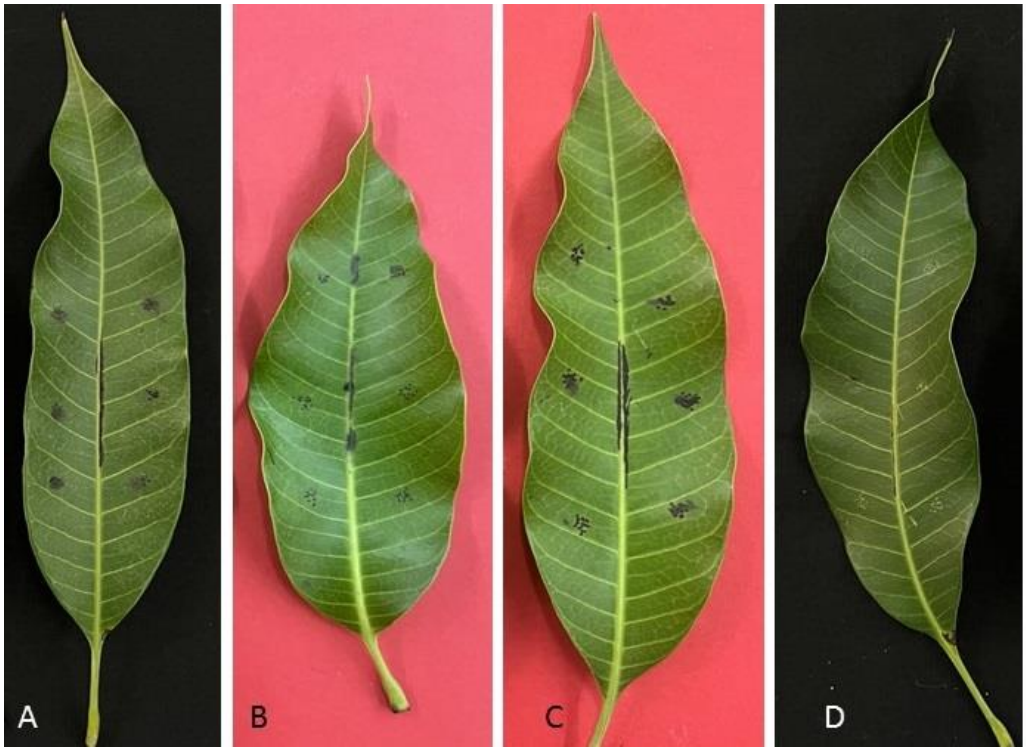

Fig. 4. Pathogenicity of obtained fungal species on leaf of mango tree seven days after inoculation, (A) *Curvularia hawaiiensis*, (B) *Exserohilum rostratum*, (C) *Alternaria alternata*, (D) Control.

Discussion

The extensive prevalence of leaf spot diseases, resulting from diverse fungal species, presents a substantial risk to mango cultivation in the south of Kerman, Sistan and Baluchestan, and Hormozgan Provinces of Iran. We surveyed leaf spot disease occurring on mango in these areas, 75 samples were obtained and one hundred and eighteen isolates were isolated. Importantly, our findings led to the isolation of *Alternaria* and *Curvularia* isolates throughout all three surveyed provinces. Additionally, the pathogenic *Exserohilum* species, was isolated from both Hormozgan and Sistan and Baluchestan Provinces.

These fungi have been reported on different hosts in various regions. For example, *Alternaria alternata* has been reported on numerous plants such as mango in Iran (Ershad, 2009). On the other hand, *E. rostratum* has previously been identified as a pathogen on cotton and gramineous plants (Sivanesan, 1987; Leonar et al., 1988; Cardona & González, 2007; Luo et al., 2012; Mirzaee et al., 2013), as well as sugarcane in Iran (Ahmadpour et al., 2013; Dokhanchi et al., 2022). Similarly, *C. hawaiiensis* has been reported on various hosts in Iran, including turfgrass, weeds, *Festuca rubra*, *Poa pratensis*, and *Agrostis tenuis* (Mirabolfathi & Ershad, 2006; Ershad, 2009).

The results of pathogenicity tests confirmed the pathogenic nature of the inoculated species including *A. alternata*, *C. hawaiiensis*, and *E. rostratum*. The complexity of fungal isolates highlights the need for a sophisticated approach to disease management, emphasizing the urgency of implementing efficient strategies to mitigate the impact of these pathogens on mango yields.

This research provides more data on distribution of *A. alternata*, *C. hawaiiensis* and *E. rostratum* fungal, the causal agents of mango leaf spot disease occurring in tropical and subtropical climate conditions of Iran.
To our knowledge, this is the first report of *C. hawaiiensis* and *E. rostratum* causing leaf spot diseases on *Mangifera indica* in Iran.

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Conflict of interest

The authors declare that there are no conflicts of interest present.

CRediT author statement

K. Dehghani: Field and laboratory works & writing original draft preparation. **A. Amirmijani**: Supervision, methodology, writing, reviewing & editing**. A. Pordel**: Supervision, methodology, writing, reviewing & editing.

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